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ANTIBODY-BASED DETECTION OF TOXINS OF BIOLOGICAL ORIGIN

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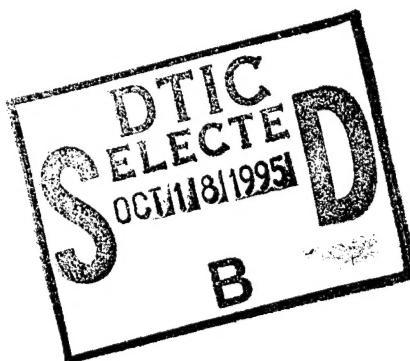
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A Fiber optic evanescent fluorosensor was used to detect the presence of toxins of biological origin. A direct competition assay was used for cholera toxin and staphylococcus entertoxin B, and an indirect competition assay for cholera, botulinum toxoid A and ricin. Detection of toxins for both methods was in the nanomolar range.

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EXECUTIVE SUMMARY

Fiber optic evanescent fluorosensors are under investigation in our laboratory for the study of drug-receptor interactions for the detection of threat agents and antibody-antigen interactions for the detection of biological toxins. In a direct competition assay, antibodies against Cholera toxin, Staphylococcus entertoxin B or ricin were noncovalently immobilized on quartz fibers and probed with fluorescein isothiocyanate labeled toxins. In the indirect competition assay, Cholera toxin or Botulinum toxoid A was immobilized onto the fiber, followed by incubation in an antiserum or partially purified anti-toxin IgG. These were then probed with fluoroscein-labeled anti-IgG antibodies. Unlabeled toxins competed with labeled toxins or anti-toxin IgG in a dose dependent manner and the detection of the toxins was in the nanomolar range. These results are compared with other methods of antibody based detection.

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PREFACE

The work described in this report was authorized under Project No. 1O162622A553. This work was started in October 1992 and completed in September 1993.

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ANTIBODY BASED DETECTION OF TOXINS OF BIOLOGICAL ORIGIN

1.   INTRODUCTION

Biomolecules bind selectively to specific target sites within their native environments. Biosensors take advantage of this natural affinity by immobilization of biological sensing elements onto various transductive devices, which convert biomolecular interactions into electrical or optical signals. Capacitance, fiber optic, ChemFET, and potentiometric devices all have been designed and tested using receptors, enzymes and antibodies as the biological sensing element.[1-4]

The fiber optic evanescent wave guide sensor detects fluorescent molecules within the evanescent wave zone which extends only a fraction ($\approx$ 1000 Å) of a wavelength above the surface of the fiber. Binding of fluorescent ligands to peptides on the surface of the fiber are easily monitored without interference from fluorescent probe in the bulk solution.

Cholera toxin (ChTX), an enterotoxin produced by Vibrio cholerae, has a molecular weight of $\approx$ 84 kD and consists of an A-subunit (MW 28 kD) noncovalently linked to five B-subunits (11.5 kD each). The B-subunit binds to receptors on the surface of the epithelial cell membrane and assists in the transport of the A- subunit through the membrane. The A-subunit catalyses the activation of adenylate cyclase which leads to electrolyte transport out of the cell and water loss resulting in severe diarrhea, dehydation and possibly death[5,6].

Staphylococcus enterotoxin B (SEB), produced by Staphylococcus aureus, is a major cause of food poisoning along with other related SE toxins. Although it is generally not fatal, the disease causes vomiting and diarrhea 4-6 hrs after ingestion of contaminated food. The preformed enterotoxins are a heterogeneous group of single chained globular toxins with molecular weights between 28-35 kD. The mechanism for poisoning is not known[7-8].

Botulism is caused by ingestion of any of the seven serologically distinguishable botulinum neurotoxins (BoTX) produced by strains of Clostridium botulinum. The toxins are large proteins (MW $\approx$ 150 kD), represented by light (53 kD) and heavy (97 kD) chains held together by a disulfide bond. Consumption of contaminated food results in muscle paralysis by depressing the release of the neurotransmitter acetylcholine. The mechanism of botulism toxicity has been suggested to be the result of a three step sequence whereby BoTX binds to acceptor in the presynaptic membrane, is transferred across the membrane to the nerve terminal where it inhibits the release of acetylcholine. Type A toxoid (Bot-A), is a detoxified aggregate form of BoTX (MW $\approx$ 500 kD)[9,10].

Ricin is a phytotoxic glycoprotein (MW 62 kD) extracted from Ricinus communis (castor bean) seeds. The toxin consists of two 31 kD subunits connected by a disulfide bond. The B-chains are lectins that bind the toxin to the cell surface and allow the A-chain to enter the cytoplasm where the eukaryotic ribosomes are inactivated, thus inhibiting protein synthesis. Ricin is highly toxic by ingestion, resulting in severe gastroenteritis, often hemorrhagic[11-13].

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Rapid detection and quantitation of toxins in biological fluids (e.g. serum) is of paramount importance in case of human and/or animal exposure. Analysis of toxins has been accomplished by means of gas chromatography/mass spectroscopy (GC/MS), latex agglutination test (LAT), radioimmunoassay (RIA) and enzyme linked immuno-sorbent assay (ELISA). Although these methods are sensitive, they are complicated, time consuming, expensive and require expert personnel.

Recent advances in optoelectronic devices have made possible the development of biosensors that are inexpensive, rapid and easy to operate. Immunosensors have been developed in our laboratory for the detection of pesticide, parathion[14] and the herbicide imazethapyr[15]. These immunosensors have many advantages over conventional immunoassays. Among the advantages is the speed of detection which represents one of the most critical factors in antidote therapy.

In the present study, we developed antibody-based immunoassays for the detection of the biological toxins cholera, staphylococcus enterotoxin B, botulinum (toxoid used for safety reasons) and ricin using the fiber optic evanescent waveguide.

2.   MATERIALS AND METHODS

2.1   Chemicals.

ChTx and goat anti-ChTx were obtained from Calbiochem (San Diego, CA). Fluoresceinated anti-goat Ab (FITC-anti-goat), SEB, rabbit anti-SEB, ricin, and FITC-ricin were obtained from Sigma Chemical Company (St. Louis, MO). BoT-A was obtained from Wako Chemicals USA, Inc. (Richmond, VA), goat anti-BoT-A antiserum was obtained from Granite Diagnostics (Burlington, NC), and goat anti-ricin was obtained from United States Army Medical Command (USAMRID), Fort Detrick, MD. All other chemicals were of the analytical grade and obtained from Sigma Chemical Company and Bio-Rad (Melville, NY).

2.2   Purification of toxins and antibodies.

Anti-ChTx and anti-BoT-A were purified by separation on a Millipore MemSep Protein G 1000 cartridge (Bedford, MA). The goat serum was loaded onto a cartridge equilibrated with binding buffer (pH 9.0) and eluted with elution buffer (pH 3.0). Eluants were monitored for absorbance at 280 nm. Fractions containing the protein were pooled, dialyzed overnight in PBS at 4°C, and were lyophilized and stored for further use.

Anti-SEB was purified by separation on an ImmunoPure Immobilized Protein A column (Pierce). The anti-SEB was loaded onto the column equilibrated with binding buffer (pH 7.5) and eluted with elution buffer (pH 2.5). Eluants were monitored for absorbance at 280 nm, and fractions containing protein were pooled and dialyzed overnight.

2.3   Detection using Evanescent Wave Sensor.

The fiber optic evanescent wave sensor, designed and built by ORD, Inc. (North Salem, NH) was used for all experimentation. Quartz fibers, 1 mm in diameter with polished ends, were obtained from ORD, Inc. The sensor makes use of the evanescent wave effect by exciting a fluorophore just outside the waveguide boundary (excitation wavelength = 485/20 nm). A portion of the resultant fluorophore emission is trapped in the waveguide and is transmitted by the fiber to the detector through 510 long pass and 530/30 nm filters. The flow cell allowed the center 47 mm of a 60 mm long fiber to be immersed in 46 μl which was exchanged every 14 sec[16].

2.4   Fluorophore labeling.

Toxin (1 mg) was reacted with 0.5 mg fluorescein isothiocyanate (FITC) on celite in 50 mM bicarbonate buffer (pH 9.5) for 30 min at room temperature. After incubation, the conjugate was briefly centrifuged to remove the celite, and then loaded onto a G-25 size exclusion column (25 x 1.1 cm) and eluted with PBS (pH 7.2). The labeled fractions were pooled and used in subsequent experiments.

2.5   Experimental protocols.

Two methods were used for toxin detection. In the first method (two step method), toxins were noncovalently adsorbed onto fibers by incubating the fibers for 2 hr in 50 μg toxin/ml of PBS, pH 7.2). After a brief rinse in PBS, the toxin-immobilized fibers were incubated 4 hr in diluted anti-toxin Ab at room temperature. Perfusion of the antibody-toxin coated fibers with fluorescein-labeled secondary antibody (0.25 μg/ml) generated an optical signal. Inhibition of antibody binding to the toxin coated fibers was achieved by incubating the fibers Ab solution containing increasing concentrations of free toxin (Figure 1).
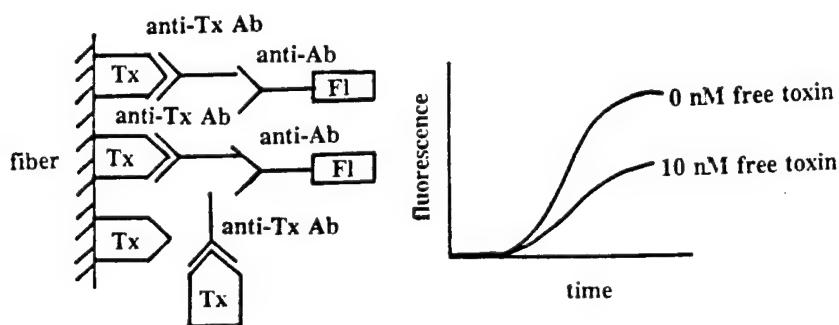

Figure 1.   Left- Schematic representation of the two step competitive inhibition of binding of fluoresceinated anti-Ab to the antitoxin Ab. Right- Simulated optical signal generated in absence and presence of 10 n toxin in incubation buffer.

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In the second method (one step method), purified anti-toxin IgG (50 μg/ml in PBS, 2 hr) was noncovalently immobilized onto the surface of the quartz fiber and probed with FITC-labeled toxin (0.5 μg/ml). Coperfusion of the antibody-coated fiber with free toxin and fluorescein-labeled toxin decreased the fluorescent signal in a dose dependent manner (Figure 2).
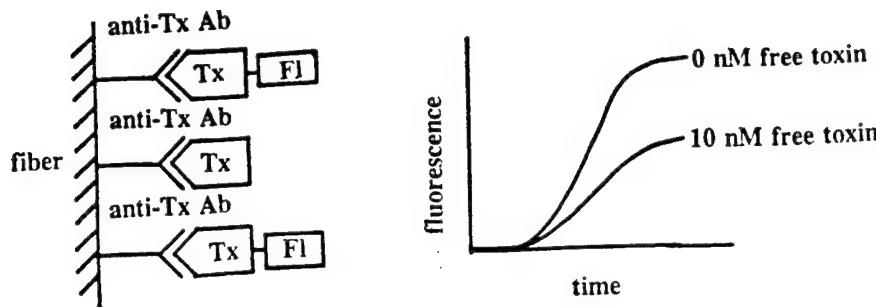

Figure 2.   Left- Schematic representation of the one step competitive inhibition of binding of fluoresceinated-toxin to Ab coated fiber. Right- Simulated optical signal generated in absence and presence of 10 nM toxin in flow buffer.

2.6   Fluorescence Measurements.

After immobilization of the toxin-antibody complex or anti-toxin Ab, the fiber was placed in the flow cell of the instrument and perfused for 5 min with perfusion solution (PBS containing 0.5% casein) to reduce the nonspecific binding[16]. The fiber was then perfused with fluorescent probe in perfusion solution. Between experiments, the flow cell was washed by flowing 1% SDS for 2 min followed by PBS for 10 min. Initial rate of fluorescence was determined graphically from the x-y record of fluorescence vs time.

3.   RESULTS

In the two step assay, incubation of toxin-coated fibers with increasing concentrations of free toxin added to antitoxin solution (0.1-100 nM) resulted in decrease of the rate of fluorescence in a dose dependent manner when probed with fluorescein-labeled secondary Ab (0.25 μg/ml). This decrease in the rate of fluorescence increase resulted from competition between the immobilized toxin on the surface of the fiber, and the free toxin added to the antitoxin-containing solution for the limited number of antibodies. Incubating the fibers in casein, rather than toxin, before soaking them in antitoxin solution generated no optical signal when the fibers were perfused with the labeled secondary Ab solution. This indicated that the Ab bound specifically to the toxin immobilized on the surface of the fiber and that the Ab did not bind to other proteins on the surface of the fiber. Flow buffer containing casein and bovine serum albumin was previously utilized to block nonspecific binding of fluorescent proteins to quartz fiber[14,16].

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When the percent rate of association (100% rate is the initial rate in the absence of free toxin) of the secondary labeled Ab to antitoxin Ab (which represented the amount of antitoxin Abs bound to the toxin coated fibers) was plotted against the concentration of free toxin, dose response curves were generated with an IC_{50} value in the nanomolar range (Figure 3).
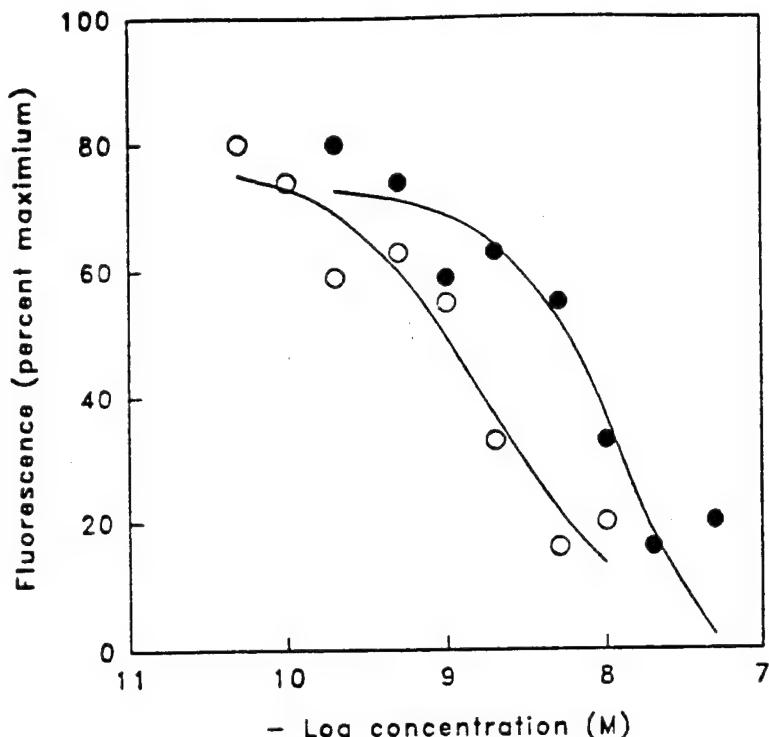
Figure 3.   Reduction in the initial rate of fluorescence resulting from competition between free and fiber-immobilized toxins for the available antitoxin Abs (two step assay). 100% is the initial rate of fluorescence resulting from binding of the secondary fluorescein-labeled Ab to fibers coated with toxin-antitoxin complex. The presence, in the incubation buffer, of increasing doses of free cholera toxin (O) or bot-A toxoid(●) decreased the rate of fluorescence in a dose dependent manner.

In the second method (one step assay), when the Ab-coated fibers were perfused with PBS/casein containing 0.25 μg/ml (perfusion solution) fluoresceinated toxin, an optical signal with high rate of fluorescence was obtained. Increasing the concentration of the free toxin in the perfusion solution decreased the fluorescent signal resulting from binding of the fluoresceinated toxin to the antibody-coated fibers. The decrease in the rate of fluorescence in the presence of free toxin correlated well with the amount of free toxin present in the flow buffer (Figure 4).
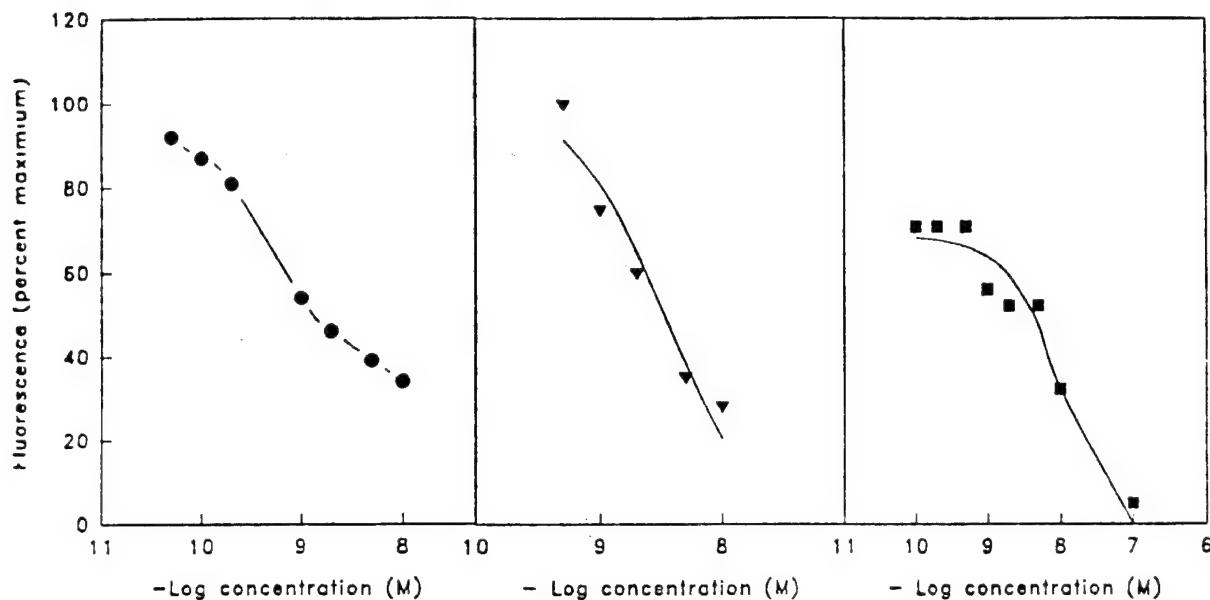
Figure 4.   Decrease in the initial rate of fluorescence resulting from competition between free and fluorescein-labeled toxin (one step assay) in the flow buffer. 100% is the initial rate of fluorescence resulting from binding of the fluoresceinated toxin to the Ab-coated fibers in the absence of free toxin. Different concentrations of free toxin (● = ricin toxin, ▼ = cholera toxin, ■ = SEB) reduced the rate of fluorescence.

The fluorescent signals generated by binding of the fluorescein-labeled toxin to antibody-coated fibers were specific as evident by the absence of measureable reduction of the optical signal when unrelated toxins were present in the flow buffer. For example, signals generated from the binding of the fluorescein-labeled cholera toxin to fiber coated with anticholera Abs were only decreased in the presence of free cholera toxin in the flow buffer, but did not show any appreciable reduction in the presence of SEB or diphtheria toxin (Table 1).

Table 1. Decrease in the rate of optical signal generated by the binding of fluoresceinated cholera toxin to fibers coated with anticholera Ab by free toxins (5 μg/ml) present in the flow buffer.

Toxin (5 μg/ml)	Percent inhibition
Cholera	72
Staphylococcus entertoxin B	0
Diphtheria	0

In both methods, (two steps and one step assay) the limit of detection was (defined as the lowest concentration of toxin that decreased the optical signal generated by the binding of toxin to its Ab) was 0.5-2 nM (0.12 ng/ml ricin, 28 ng/ml SEB, 42 ng/ml ChTx two step, 84 ng/ml ChTx one step, 1000 ng/ml Bot-A), while the IC_{50} values were 10-50 nM.

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4.   DISCUSSION

Biosensors are extremely useful analytical devices for detection and quantitation of drugs[16], toxins and chemical pollutants[15]. Among these advantages are portability, speed of detection, simplicity, cost effectiveness and selectivity and sensitivity. Most equipment used for analytical purposes is designed to operate in laboratories. Biosensors, however, are more suitable to field, clinics and personal use (e.g. glucose sensor). The biosensor used in these studies is no larger than a small briefcase and could easily be miniaturized to a handheld device that would use batteries to operate and would give a direct digital readout. In case of exposure or emergency medical situation, speed is of primary importance for life saving and biosensors are excellent tools for speedy diagnosis and identification of a causative factor. The assay can be performed in minutes to provide the patient with results of analysis while still in the clinic.

The operation of the biosensor is extremely simple, involving the insertion of an optic fiber and turning the perfusion pump on and off. The time required for training can be as little as 2 hrs. Moreover, automation could further reduce training time to a few minutes. The cost of the assay is a few dollars and could be reduced by new production technology, use of cheap disposable kits, regenerable biosensors, simplification of the assay, and a large enough market to increase production and reduce the cost. In case of suspected exposure of humans and/or animals to certain toxins, it is of paramount importance to have a sensitive and extremely specific assay. The progression of the disease state in case of poisoning is often rapid. Early and rapid recognition of the causative agent and aggressive antidote therapy are the keys to successful management of patients exposed to noxious agents.

Sensitivity and selectivity depend, to a large degree, on the properties of the immobilized Ab, rather than the physical transducer. The higher the affinity of the Ab immobilized on the surface of the fiber to the antigen, the greater sensitivity of the sensor. In our studies, detection limits of most toxins compared well with the sensitivity of other analytical techniques. A high affinity mAb would increase the sensitivity of the sensor manyfold. The fiber optic immunosensor that was used in this study exhibits many of the forementioned advantages. One major advantage of the fiber optic fluorosensors over other analytical techniques is their ability to detect the analyte of interest in crude samples[15]. This is possible because it is the fluorescence that is measured and other materials in the sample including colors would not interfere with the measurement. Since the evanescent wave extends about 100 nm, only binding events on the surface of the fiber can be detected. In other words, the sensor is blind to chemicals in the bulk solution. This property is critical for rapid detection and field use.

The toxin immunosensor described in the present studies detects toxins in the nanomolar range. This makes the immunosensor a very sensitive analytical device for toxin detection and quantitation. The sensor described in this study was also extremely selective and showed no cross reactivity between different toxins. Of the two methods used for toxin detection, the two step assay was more sensitive. However, the one step competition assay was simpler and less time consuming.

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Immunological methods have been developed for quantitation of antibodies and antigens. These methods require some sample cleanup, and are labor intensive, expensive, or requiring multiple steps. The limit of detection (nanomolar range) of these immunoassays are compared in Tables 2-5.

Table 2. Antibody-based detection of botulinum toxin

TECHNIQUE	SENSITIVITY
1. Fiber Optic Waveguide (FOWG) (toxoid)	1000 ng/ml(Figure 2)
2. FOWG	<300 ng[17]
3. Fiber Optic Biosensor	5 ng/ml[18]
4. Enzyme Linked Immunosorbent Assay (ELISA)	5-10 pg/ml[19]
5. Chemiluminescent Linked Immunosorbent Assay (CLISA)	1 pg/ml[20]
6. Enzyme Linked Coagulation Assay (ELCA)	<10 pg/ml[21]
7. Light Addressable Potentiometric Sensor (LAPS) (toxoid)	2 ng/ml[22]
8. ORIGEN Electrochemiluminescence Detector (toxoid)	25 fg/ml[23]

Table 3. Antibody-based detection of cholera toxin.

TECHNIQUE	SENSITIVITY
1. FOWG (2 step)	42 ng/ml (Figure 2)
2. FOWG (1 step)	84 ng/ml (Figure 3)
3. Bead enhanced ELISA	40 pg/ml[24]
4. Bead Enhanced ELISA	26 pg/ml[25]
5. LAPS	2 ng/ml[22]
6. ORIGEN	25 fg/ml[23]

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Table 4. Antibody-based detection of SEB

TECHNIQUE	SENSITIVITY
1. FOWG	28 ng/ml
2. ELISA	0.5-0.75 ng/ml[26]
3. ELISA/Enzme Linked Fluorometric Assay	1-2 ng/ml[27]
4. Fluorogenic Enzyme Linked Immunosorbent Assay (FELISA)	0.1 fg/ml[28]
5. LAPS	2 ng/ml[22]
6. ORIGEN	25 fg/ml[23]
7. ELISA	0.5 ng/ml[29]
8. ELISA	0.5-1 ng/ml[30]
9. ELISA	0.5-1 ng/ml[31]

Table 5. Antibody-based detection of ricin.

TECHNIQUE	SENSITIVITY
1. FOWG	0.3 ng/ml
2. LAPS	10 ng/ml[22]
3. ORIGEN	250 fg/ml[23]
4. ELISA	0.1-1 ng/ml[32]
5. ELISA	0.002 ppm[33]
6. Fiber Optic Biosensor	1 ng/ml[34]
7. ELISA	500 pg/ml[34]

In summary, the fiber optic system used in this study provides a rapid, inexpensive and equally sensitive alternative assay that is not affected by crude samples or electrostatic interference.

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